



## Original Contribution

Pro-inflammatory Macrophages suppress PPAR $\gamma$  activity in Adipocytes via S-nitrosylation

Ruiying Yin<sup>a</sup>, Li Fang<sup>a</sup>, Yingjia Li<sup>a</sup>, Peng Xue<sup>b</sup>, Yazhi Li<sup>b</sup>, Youfei Guan<sup>c</sup>, Yongsheng Chang<sup>d</sup>, Chang Chen<sup>b,\*</sup>, Nanping Wang<sup>a,c,\*</sup>

<sup>a</sup> Institute of Cardiovascular Science and Diabetes Center, Peking University, Beijing 100191, China

<sup>b</sup> Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

<sup>c</sup> The Advanced Institute for Medical Sciences, Dalian Medical University, Dalian 116044, China

<sup>d</sup> National Laboratory of Medical Molecular Biology, Institute of Basic Medical Science, Chinese Academy of Medical Science and Peking Union Medical College, Beijing 100005, China

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## ABSTRACT

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is a ligand-activated nuclear receptor and plays an essential role in insulin signaling. Macrophage infiltration into adipose tissue is a character of metabolic inflammation and closely related to insulin resistance in type 2 diabetes. The mechanism by which pro-inflammatory macrophages cause insulin resistance remains to be elucidated. Here we showed that co-culture with macrophages significantly suppressed the transcriptional activity of PPAR $\gamma$  on its target genes in 3T3-L1 preadipocytes and diabetic primary adipocytes, depending on inducible nitric oxide synthase (iNOS). We further showed that PPAR $\gamma$  underwent S-nitrosylation in response to nitrosative stress. Mass-spectrometry and site-directed mutagenesis revealed that S-nitrosylation at cysteine 168 was responsible for the impairment of PPAR $\gamma$  function. Extended exposure to NO instigated the proteasome-dependent degradation of PPAR $\gamma$ . Consistently, *in vivo* evidence revealed an association of the decreased PPAR $\gamma$  protein level with increased macrophage infiltration in visceral adipose tissue (VAT) of obese diabetic *db/db* mice. Together, our results demonstrated that pro-inflammatory macrophages suppressed PPAR $\gamma$  activity in adipocytes via S-nitrosylation, suggesting a novel mechanism linking metabolic inflammation with insulin resistance.

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## 1. Introduction

Adipose tissue is an important organ that maintains the homeostasis of glucose and lipid metabolism. In addition to the function as an energy storage tissue, adipose tissue secretes a series of adipokines including adiponectin to regulate glucose and lipid metabolism in other insulin sensitive tissues such as liver and skeletal muscles [1]. In obesity and other metabolic disorders, the uptake of excessive nutrients and energy causes a metabolic surplus, leading to a chronic low-grade inflammation status called metabolic inflammation or meta-inflammation [2]. In adipose tissues of obese and diabetic animals and patients, the metabolic inflammation is featured with the infiltration and activation of macrophages, impaired insulin sensitivity and dysfunctional secretion of adipokines [3–5].

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), a member of nuclear receptor superfamily, is highly expressed in adipose tissues. PPAR $\gamma$  is a master regulator of glucose and lipid metabolism [6,7]. PPAR $\gamma$  heterodimerizes with retinoid X receptor- $\alpha$ , binds to specific PPAR-responsive elements (PPRE) in the target genes, and leads to their transcriptional activations upon the ligand binding [8]. Thiazolidinediones (TZDs) including rosiglitazone and pioglitazone are synthetic ligands selective for PPAR $\gamma$  and have been widely used in the treatment of type 2 diabetes mellitus as insulin sensitizers [9,10]. The insulin sensitizing effects of TZDs are predominantly mediated by the actions of PPAR $\gamma$  in adipose tissues via at least the two mechanisms: 1) promoting the uptake of glucose and free fatty acids for adipogenesis, and therefore decreasing their levels in circulation; aP2 (fatty acid-binding protein 4, FABP4) [11], stearoyl-CoA desaturase (SCD1) [12], acyl-CoA synthetase, and perilipin [13] are among the PPAR $\gamma$  target genes responsible for this mechanism; 2) inducing the expression of adipokine genes such as adiponectin to improve the insulin signaling in other organs [14,15]. In expanding adipose

\* Corresponding authors.

E-mail addresses: [changchen@moon.ibp.ac.cn](mailto:changchen@moon.ibp.ac.cn) (C. Chen), [nanpingwang2003@yahoo.com](mailto:nanpingwang2003@yahoo.com) (N. Wang).

tissue, monocytes and macrophages are recruited and activated, exhibiting pro-inflammatory features. These pro-inflammatory (classically activated) macrophages release a series of pro-inflammatory factors like TNF $\alpha$ , IL-1 $\beta$ , IL-6 and nitric oxide (NO). Among them, excessive NO derived from inducible NO synthase (iNOS) acts with reactive oxidative species (ROS) to produce a nitrosative stress [16] which may impair adipocyte function [17,18]. Nitrosative stress can trigger a redox sensitive process called protein S-nitrosylation [19], i.e. the reaction of thiols at the cysteine residues in the substrate proteins with NO or NO-derived species, forming -SNO group [20]. S-nitrosylation is an important mechanism by which NO exerts its physiological and pathological functions [21,22]. Previous studies indicated that macrophages inhibited adipocyte differentiation via a paracrine action [23]. In addition, NO donor blunted PPAR $\gamma$  activity in 3T3-L1 preadipocytes [24]. However, the molecular mechanism by which NO suppresses PPAR $\gamma$  function remains unknown.

In the present study, we found that pro-inflammatory macrophages instigated the S-nitrosylation of PPAR $\gamma$  in adipocytes and impaired the transcriptional activity and protein stability of PPAR $\gamma$ . This finding suggested a novel mechanism linking metabolic inflammation with insulin resistance.

## 2. Research Design and Methods

### 2.1. Reagents and Chemicals

Polyclonal rabbit anti-PPAR $\gamma$  antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat monoclonal anti-F4/80 antibody was from Abcam (Cambridge, MA, USA). S-Nitrosoglutathione (GSNO) was synthesized with glutathione (GSH) and sodium nitrite. Rosiglitazone, MG132, lipopolysaccharide (LPS), 1400 W, ethylene diamine tetraacetic acid (EDTA), isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), sodium ascorbate, N-acetylcysteine (NAC), neocuproine, methylmethanethiosulfonate (MMTS), dithiothreitol (DTT), biotin-maleimide, streptavidin-agarose, protein A/G agarose, insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and Oil Red O were purchased from Sigma (St. Louis, MO, USA). MTS solution was purchased from Promega (San Luis Obispo, CA, USA). Restriction endonucleases and other modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA). 0.4  $\mu$ m Transwell™ Permeable Support was purchased from Corning (Tewksbury, MA, USA).

### 2.2. Animal Procedure

Male Sprague-Dawley rats at 6 weeks age and male *db/m* or *db/db* mice at 5 or 16 weeks age were used. Experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals with the approval by the institutional committee. Rats and mice were euthanized by terminal bleeding under anesthesia (pentobarbital sodium, 60 mg/kg).

### 2.3. Cell culture

3T3-L1, Raw264.7 and HeLa cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Primary preadipocytes were isolated from rat epididymal adipose tissue and grown in 1:1 F12/DMEM supplemented with 10% FBS. In the co-culture system, Raw264.7 cells were washed after indicated treatments and seeded in the upper chamber, while 3T3-L1 cells or differentiated

primary preadipocytes (hereinafter referred to as primary adipocytes) were grown in the lower chamber.

### 2.4. Plasmids Construction and Transfection

PPRE-TK-Luc, pRSV-gal and CMX-mPPAR $\gamma$ 1 encoding a murine PPAR $\gamma$ 1 were described [25]. Site-directed mutations were generated using QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), with primers containing -GCC- (coding alanine) mutation instead of original -TGC- (coding cysteine). GST-PPAR $\gamma$  was constructed through subcloning of the mouse PPAR $\gamma$ 1 coding region from CMX-mPPAR $\gamma$ 1 into pGEX-6 P-1 vector. Plasmids were transfected using Lipofectamine 2000 (Life Technologies).

### 2.5. Adenoviral Infection

3T3-L1 or HeLa cells were co-infected with Ad-VP-PPAR $\gamma$  or Ad-PPAR $\gamma$  that encodes mouse or human PPAR $\gamma$ 1, and Ad-tTA that encodes a tetracycline-responsive transactivator (a *tet-off* system) as described [25]. Then cells were maintained in the presence or absence of tetracycline (0.1  $\mu$ g/mL) for the indicated time.

### 2.6. Detection of S-nitrosylation by irreversible biotinylation procedure (IBP)

IBP was performed as previously described [26]. Briefly, cells were homogenized in RIPA lysis buffer. After centrifugation, MMTS (20 mM) was added to the supernatant and incubated at 50 °C for 30 min to block free thiols. After acetone precipitation and centrifugation, the pellet was recovered in HENS buffer containing 0.2 mM biotin-maleimide with 10 mM ascorbate and incubated at 37 °C for 1 h. Then, excess biotin-maleimide was removed by ice-cold acetone precipitation. The samples were recovered and boiled in HENS buffer containing 200 mM DTT for 15 min to reduce potential intermolecular disulfide bonds. After neutralization, the biotinylated protein was purified with streptavidin-agarose and eluted with HENS buffer. Samples were analyzed with Western blotting to detect S-nitrosylated PPAR $\gamma$ .

### 2.7. Western blotting

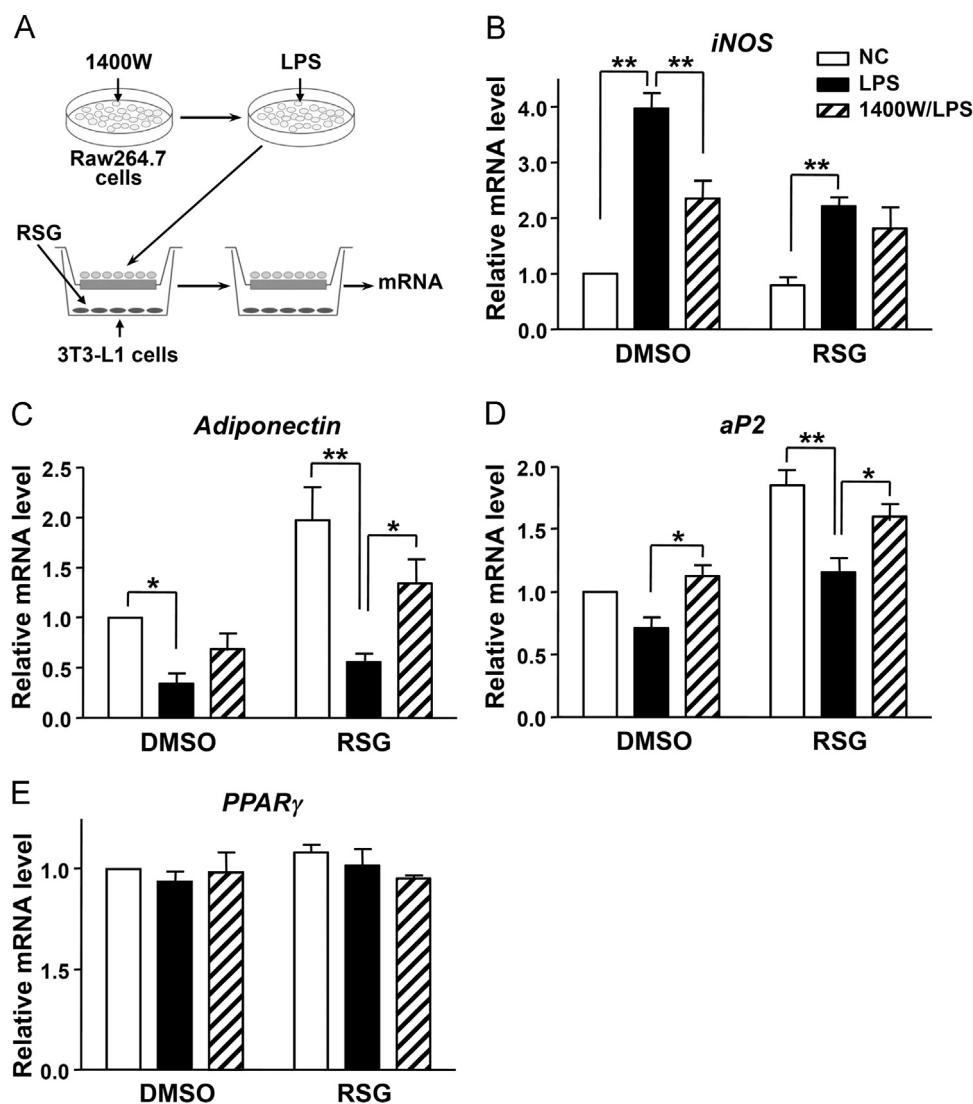
Protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gels electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween (0.2%) (TBS-T) for 1 h, incubated overnight with primary antibody of PPAR $\gamma$  (1:1000 dilution in blocking buffer) and then horseradish peroxidase (HRP)-conjugated secondary antibody. After visualizing with enhanced chemiluminescence (ECL) reagent, autoradiographs were scanned or exposed to X-ray films and quantified for band intensities.

### 2.8. Quantitative reverse-transcriptase-PCR (qRT-PCR)

Total RNA was isolated from cells or tissues with Trizol Reagent (Life Technologies) and reverse transcribed by using M-MLV transcriptase and random primers. qPCR was performed with the GoTaq® qPCR Master Mix (Promega) and specific primers (sequences in Supplementary Table 1) in the Stratagene Mx3000P qPCR System (Agilent Technologies).

### 2.9. Chromatin Immunoprecipitation (ChIP) Assay

Cells were cross-linked with 1% formaldehyde for 10 min, harvested and subjected to sonication (25 W, 15 s  $\times$  3). The



**Fig. 1.** Repression of PPAR $\gamma$  target genes in 3T3-L1 cells by NO derived from Raw264.7 macrophages. Raw264.7 cells were stimulated with LPS (100 ng/mL, 16 h), with or without 1400 W pretreatment (10  $\mu$ M, 2 h). 3T3-L1 cells were co-cultured with Raw264.7 cells for 4 h and then treated with rosiglitazone (RSG, 10  $\mu$ M, 24 h) or DMSO (A). The mRNA levels of *iNOS* in Raw264.7 cells (B) and *adiponectin* (C), *aP2* (D) and *PPAR $\gamma$*  (E) in 3T3-L1 cells were analyzed by using qRT-PCR. Data were means  $\pm$  SEM from 3 independent experiments. Statistical comparisons were made with two-way ANOVA. \* $p$  < 0.05, \*\* $p$  < 0.01.

sheared chromatin was then immunoprecipitated with anti-PPAR $\gamma$  antibody or control IgG (2  $\mu$ g for each sample) and pulled-down by protein A/G agarose beads. After washing and eluting, immunocomplexes were reversed in 65  $^{\circ}$ C. DNA was purified by using the QIAquick<sup>®</sup> PCR purification kit (QIAGEN), followed by qPCR with the primers (Supplementary Table 1) flanking the putative PPRE motifs in *aP2* and *adiponectin* promoter regions.

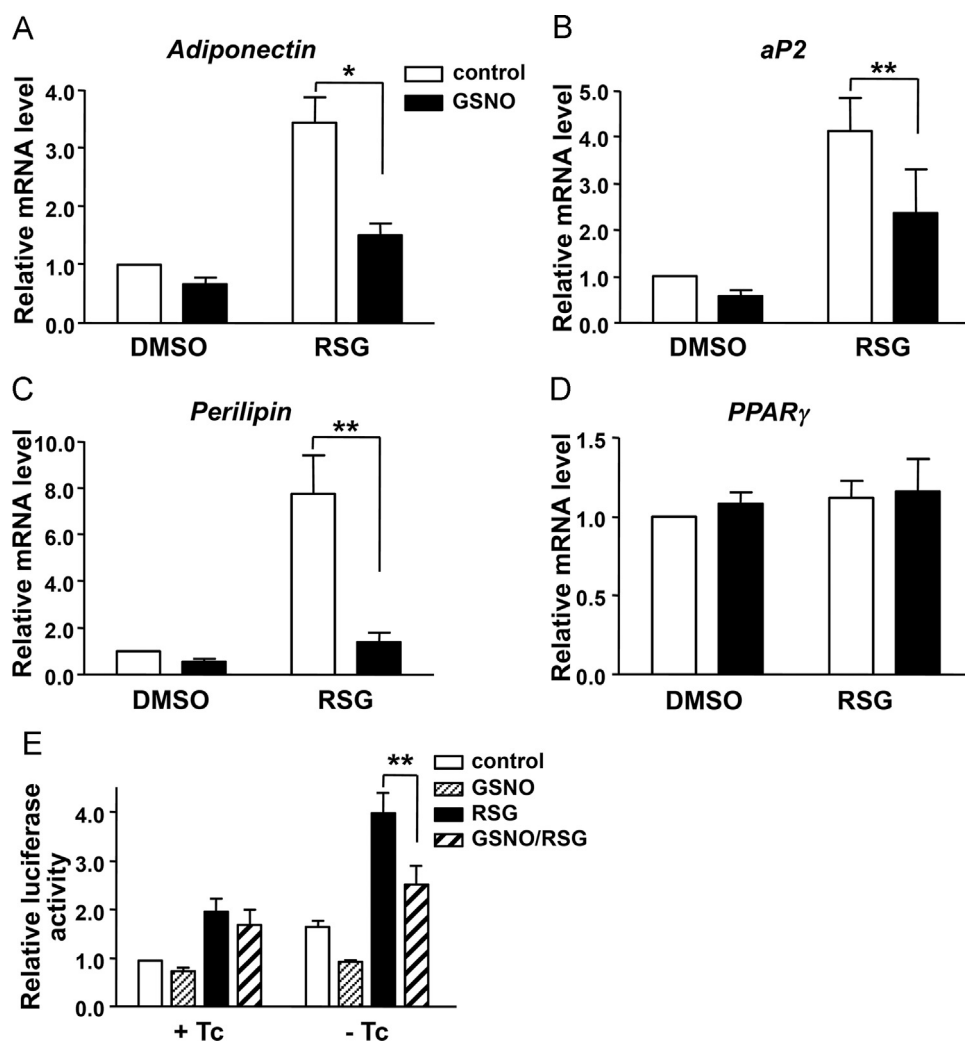
#### 2.10. Expression and purification of recombinant PPAR $\gamma$ protein

BL21 ( $\lambda$ DE3) cells were transformed with GST-mPPAR $\gamma$ 1 and induced to express recombinant PPAR $\gamma$  by IPTG (0.5 mM, 16  $^{\circ}$ C, 16 h). Cells were harvested in TBS buffer containing DTT (5 mM), phenylmethylsulfonyl fluoride (1 mM) and EDTA (1 mM). After centrifuging, the supernatant was sonicated and loaded to the glutathione-Sepharose column (National Engineering Research Center for Biomaterials, NERC). The column was washed with TBS for three times and the protein was eluted with TBS buffer containing GSH (20 mM). The purified protein was dialyzed against TBS buffer containing DTT (25  $\mu$ M) to remove GSH and maintain the activity of the protein.

#### 2.11. Mass spectrometry determination of S-nitrosylated sites

Purified GST-mPPAR $\gamma$ 1 protein (200  $\mu$ g) was treated with 1 mM GSNO for 30 min at room temperature. S-nitrosylated proteins were blocked by MMTS and labeled by biotin-maleimide as described in IBP. Biotinylated proteins were then trypsinized (using a 1:50 ratio of protein: trypsin) in solution for 16 h and purified with streptavidin-agarose for liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Peptides were separated by the NanoLC 1D Plus system (RP-C18, 0.15 mm  $\times$  2.5 mm trapping columns and 0.075 mm  $\times$  200 mm analytical columns) at a flow rate of 300 nL/min. Eluting peptide cations were ionized by nanospray and analyzed by a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). A survey full scan followed by 10 CID events was used. Dynamic exclusion for selected precursor ions was set at 120 s.

MS/MS spectra were searched against UniProt mouse proteome database (Release 2013\_06) using SequestHT. Database searches were performed with the following parameters: precursor mass tolerance 10 ppm; MS/MS mass tolerance 0.8 Da; two missed cleavage for trypsinized peptides; variable modifications oxidation (M, +15.9949 Da), methylthio (C, +45.9877 Da), biotin-maleimide



**Fig. 2.** Suppression of PPAR $\gamma$  transcriptional activity by GSNO. (A–D) After pretreatment with GSNO (1 mM, 4 h), 3T3-L1 cells were stimulated with RSG (10  $\mu$ M, 24 h). The mRNA levels of *adiponectin*, *aP2*, *perilipin* and *PPAR $\gamma$*  were analyzed with qRT-PCR. (E) HeLa cells were infected with Ad-PPAR $\gamma$  in the presence or absence of tetracycline (Tc, 1  $\mu$ g/mL, tet-off) and then transfected with PPRE-TK-Luc and pRSV- $\beta$ -gal. Cells were exposed to GSNO (4 h) and stimulated with RSG (24 h). Bar graph represents the luciferase activity after normalization to  $\beta$ -galactosidase activity. \* $p < 0.05$ , \*\* $p < 0.01$ .

(C, +451.1889 Da). The results were filtered for a 1% false discovery rate (FDR) at the PSM level utilizing the percolator-based algorithm [27].

## 2.12. Isolation of rat primary preadipocytes

Epididymal fat pads of Sprague-Dawley rats, or 5-week old *db/m* or *db/db* mice were isolated, washed in phenol red-free DMEM, and cut into pieces less than 1 mm<sup>3</sup>. Collagenase (1 mg/mL) was used to digest the tissue in a shaking water bath (37 °C, 40 min, 120 rpm). The mixture was diluted with phenol red-free DMEM (5 mL) and filtrated with a 100-mesh cellcribble. After standing for 10 min, the suspensions below the float were transferred. The floating components were washed with phenol red-free DMEM for three times. The lower suspensions were collected and filtrated with a 400-mesh cellcribble. Then, the irrelevant components were removed through pre-centrifuge (160 g, 5 min). After centrifuge at 800 g for 10 min, preadipocytes were obtained, resuspended and cultured in F12/DMEM (1:1) containing 10% FBS.

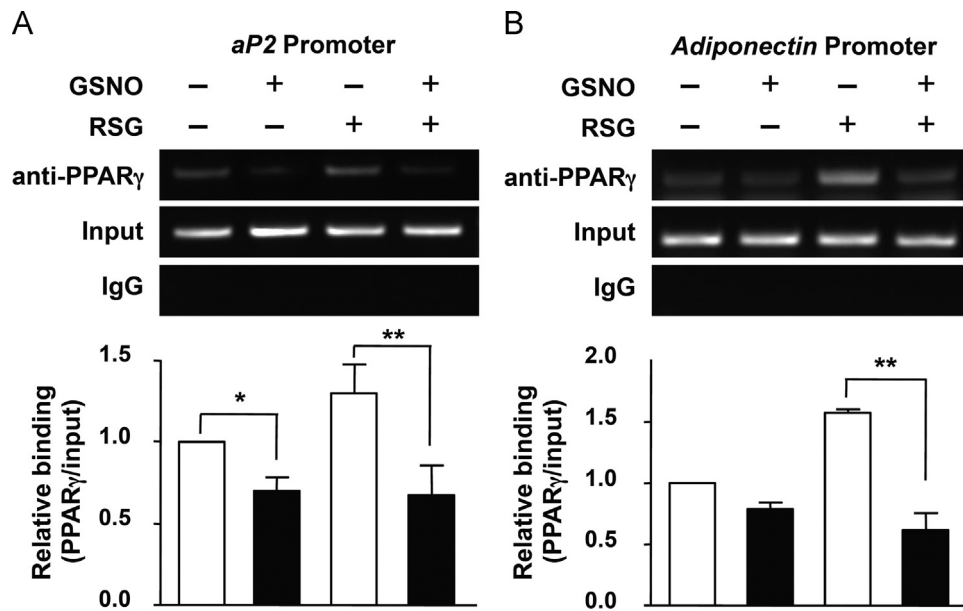
## 2.13. Adipocyte differentiation

After confluence, primary preadipocytes were kept for another 2 days and then treated with adipocyte differentiation media

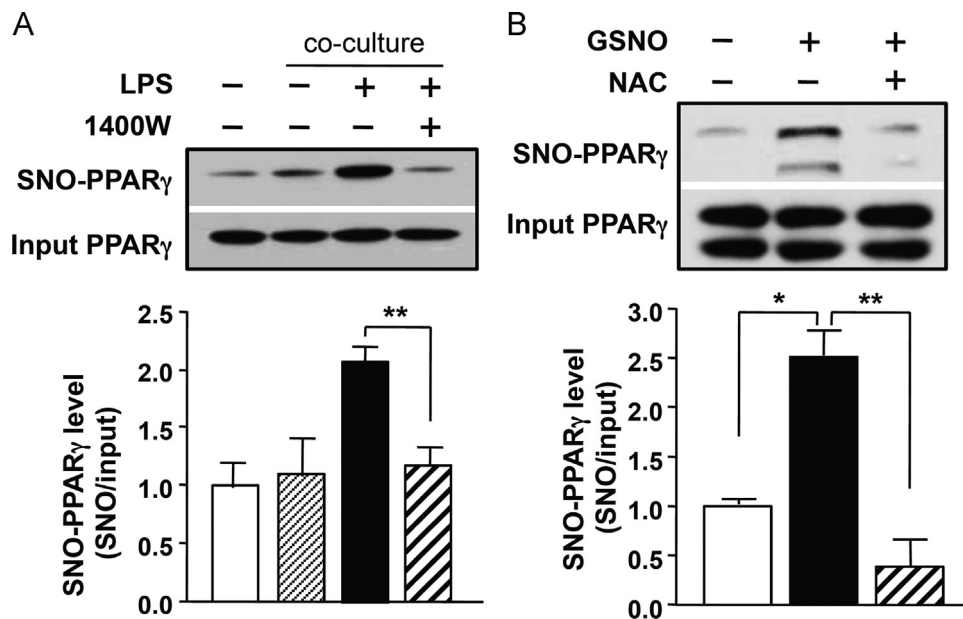
(1  $\mu$ M dexamethasone, 0.5 mM IBMX, 10  $\mu$ g/mL insulin in complete F12/DMEM media). After 3 days, cells were switched to the maturation media (10  $\mu$ g/mL insulin in complete F12/DMEM media) for 2 days. Then, the media were half-changed with F12/DMEM every 2 days thereafter. Adipogenic differentiation was evaluated with Oil Red O staining. Briefly, cells were fixed with 4% paraformaldehyde (PFA) for 30 min, washed, and then stained with fresh Oil Red O (0.3%, in 60% isopropanol) for 1 h. Stained cells were photographed by Leica DMI 3000B system.

## 2.14. Immunohistochemistry Staining

Paraffin sections from epididymal fat tissue were dewaxed and pretreated with 3% H<sub>2</sub>O<sub>2</sub> in methanol to inactivated endogenous peroxidase. After antigen retrieval in EDTA (pH 8.0) buffer, tissue sections were blocked by goat serum (10%) for 1 h and incubated with the rat antibody against F4/80 at 4 °C overnight. Then the sections were incubated with HRP-conjugated goat anti-rat IgG antibody, developed with 3,3'-diaminobenzidine (DAB) chromogen substrate and counterstained with hematoxylin. Images were captured by Leica DM 3000 system.



**Fig. 3.** Decrease of the PPAR $\gamma$  DNA binding by GSNO. 3T3-L1 cells were treated with GSNO (1 mM, 4 h) and then stimulated with RSG (10  $\mu$ M, 24 h). ChIP assays were performed with PPAR $\gamma$  antibody and primers flanking the PPRE motifs in the *aP2* and *adiponectin* promoter regions. PCR amplification products of *aP2* (A) and *adiponectin* (B) promoter were resolved on agarose gel. Relative enrichment of PPAR $\gamma$  was expressed by fold change above input group.



**Fig. 4.** Detection of S-nitrosylation of PPAR $\gamma$  with IBP. (A) 3T3-L1 cells were co-cultured with the LPS pretreated Raw264.7 (with or without 1400 W) for 4 h. Total proteins were extracted from 3T3-L1 cells and subjected to IBP detection of S-nitrosylated PPAR $\gamma$ . (B) 3T3-L1 cells were infected with Ad-VP-PPAR $\gamma$  for 24 h and exposed to GSNO (1 mM for 4 h) with or without NAC pretreatment (10 mM, 2 h). The bar graphs are mean  $\pm$  SEM of 3 independent experiments.

#### 2.15. MTS Assay

Raw264.7 cells, 3T3-L1 cells or HeLa cells were seeded in 96-well plate with  $5 \times 10^3$  cells per well. After 1400 W or GSNO treatment for indicated times, MTS solution was added to the medium and cells were incubated for 1 hour. Cell viability was assessed by OD values at 490 nm.

#### 2.16. Statistical analysis

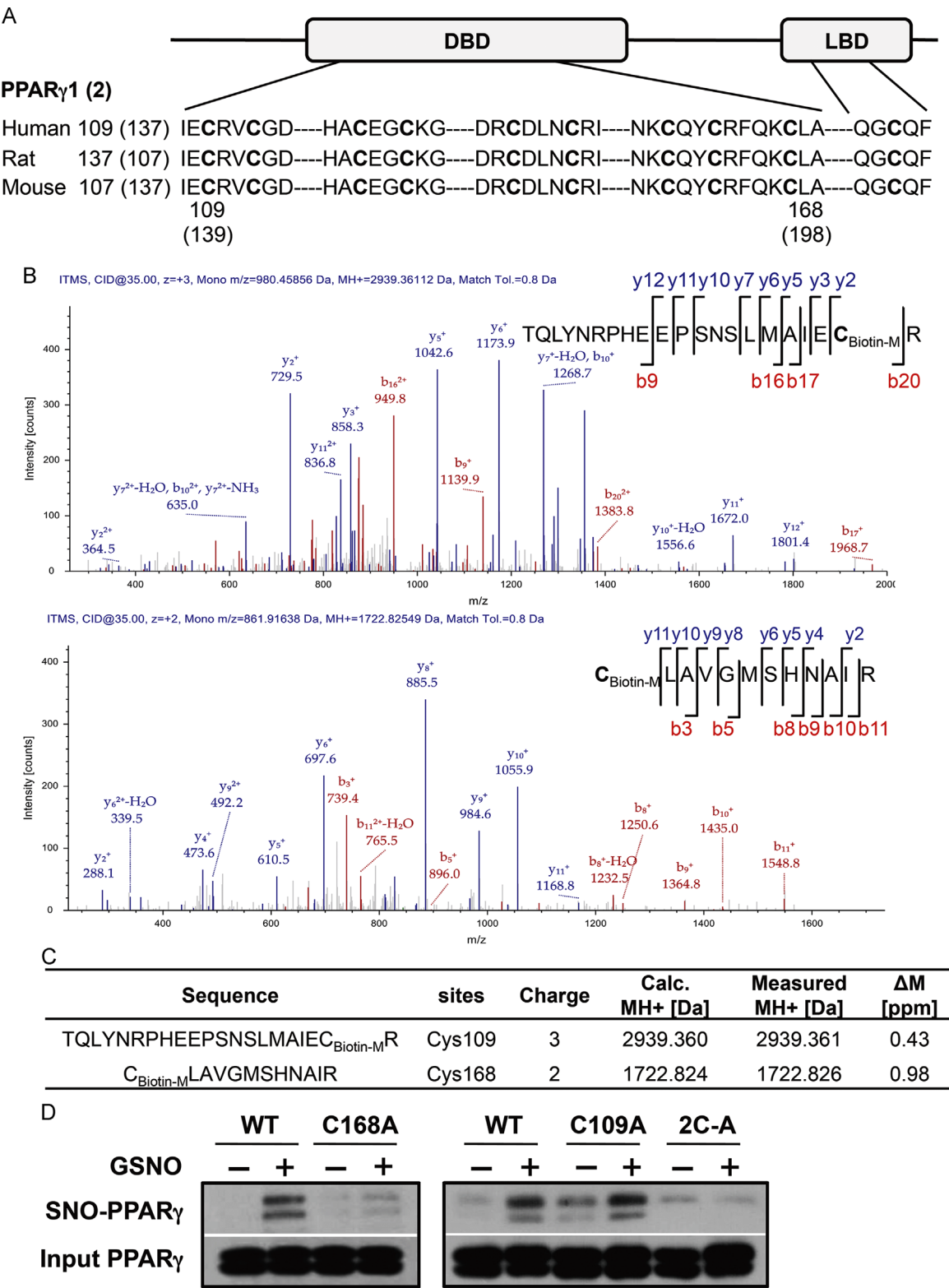
Quantitative results were expressed as means  $\pm$  SEM. Student's *t* test between two groups or one/two-way ANOVA followed by Tukey's test (multigroup comparisons) were used to analyze the statistical differences (GraphPad Prism, version 6.0, GraphPad).

$p < 0.05$  was considered significant. Non-quantitative results were representative of at least 3 independent experiments.

### 3. Results

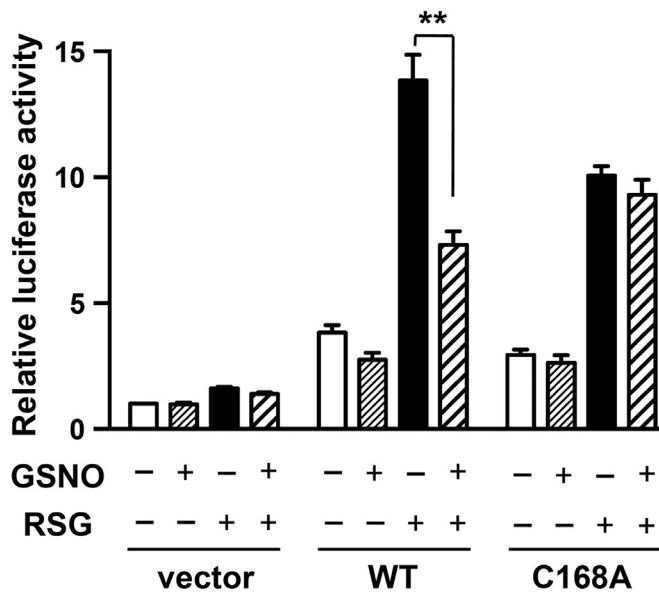
#### 3.1. Macrophages-derived NO suppressed PPAR $\gamma$ target genes in preadipocytes

In order to examine the crosstalk between the pro-inflammatory macrophages and adipocytes, we co-cultured 3T3-L1 preadipocyte with Raw264.7, a macrophage cell line (Fig. 1A). Prior to the co-culture, Raw264.7 were stimulated with LPS to induce the expression of iNOS (Fig. 1B). Incubation with the LPS-



**Fig. 5.** Identification of S-nitrosylated cysteine residues in PPAR $\gamma$ . (A) Alignment of cysteine residues (bolded) among human, rat and mouse PPAR $\gamma$ 1 proteins. The numbers in the parentheses indicate the corresponding positions in PPAR $\gamma$ 2. (B) S-nitrosylation of GST-mPPAR $\gamma$ 1 was detected by using LC-MS/MS. Sequence-informative fragmentation ions were summarized on the peptide sequence and annotated in red (b-ions) and blue (y-ions). (C) Biotin-maleimide (Biotin-M) labeled (S-nitrosylated) peptides identified by LC-MS/MS in order of confidence. The number of spectra identified for each S-nitrosylated peptide was listed, along with the calculated (Calc.) and measured mono-isotopic masses of the peptides ( $[M+H]^+$ ) and the accuracy of the mass measurements in parts per million (p.p.m.). (D) HeLa cells were transfected with the PPAR $\gamma$ 1 mutants (C168A, C109A or 2 C-A) and, 24 h later, treated with GSNO. S-nitrosylated PPAR $\gamma$  was analyzed by using IBP followed by Western blotting.

stimulated Raw264.7 significantly suppressed the induction of PPAR $\gamma$  target genes *adiponectin* and *aP2* by rosiglitazone in 3T3-L1 cells (Fig. 1C-D). Pretreatment of Raw264.7 with 1400 W, a selective inhibitor of iNOS, attenuated the suppressive effects of the Raw264.7 on the PPAR $\gamma$  target genes. The treatment with 1400 W did not affect cell viability (Supplementary Fig. 1A).



**Fig. 6.** Mutation of Cys168 reduced the suppression of PPAR $\gamma$  activity by GSNO. Wild type (WT) or mutant PPAR $\gamma$  (C168A) was co-transfected into HeLa cells with PPRE-TK-Luc. After 24 h, cells were pretreated with GSNO for 4 h and then stimulated with RSG for 24 h. Relative luciferase activities were shown as the fold induction. \*\* $p < 0.01$ .

Interestingly, co-culture with Raw264.7 macrophages did not reduce the expression level of PPAR $\gamma$  in 3T3-L1 cells (Fig. 1E). These results suggested that pro-inflammatory macrophage-derived NO suppressed the induction of PPAR $\gamma$  target genes in preadipocytes, likely via a negative regulation of the transactivation capacity of PPAR $\gamma$ .

### 3.2. NO donor negatively regulated PPAR $\gamma$ transcriptional activity

To examine the suppressive effects of NO on PPAR $\gamma$  transcriptional activity, 3T3-L1 cells were treated with NO donor GSNO (1 mM, 4 h). In Fig. 2A–C, qRT-PCR showed that the induction of *adiponectin*, *ap2* and *perilipin* mRNA by rosiglitazone were significantly decreased by GSNO. GSNO had little effect on PPAR $\gamma$  gene expression (Fig. 2D) and cell viability (Supplementary Fig. 1B). Next, we performed luciferase reporter assay to examine the regulation of PPAR $\gamma$  transcriptional activity by GSNO. HeLa cells were used because it lacks endogenous PPAR $\gamma$  expression [28]. After adenovirus-mediated overexpression of PPAR $\gamma$ , cells were transfected with PPRE-TK-Luc and treated with GSNO. GSNO did not decrease HeLa cells viability significantly (Supplementary Fig. 1C). As shown in Fig. 2E, GSNO decreased the reporter activity and the activation by rosiglitazone, indicating that the transactivation capacity of PPAR $\gamma$  was compromised by the NO donor.

ChIP assay was performed to explore whether the suppression of PPAR $\gamma$  activity by NO was due to a decreased binding to the promoters of its target genes. As shown in Fig. 3, GSNO significantly reduced the rosiglitazone-stimulated enrichment of PPAR $\gamma$  on the cognate DNA elements within the murine *ap2* and *adiponectin* promoter regions. Thus, inhibition of the PPAR $\gamma$  DNA-binding capacity by NO might account for the suppression of PPAR $\gamma$  activity.

### 3.3. PPAR $\gamma$ was modulated by S-nitrosylation

We examined whether NO negatively regulated PPAR $\gamma$  activity by S-nitrosylation. IBP showed that co-culture with LPS-stimulated Raw264.7 macrophage increased S-nitrosylation of PPAR $\gamma$  in 3T3-L1 cells. The iNOS inhibitor 1400 W reduced the PPAR $\gamma$

S-nitrosylation (Fig. 4A). Similarly, GSNO treatment also increased PPAR $\gamma$  S-nitrosylation. NAC, a compound known to promote de-nitrosylation, abrogated the GSNO-induced S-nitrosylation (Fig. 4B).

Examination of the protein sequence of PPAR $\gamma$ 1 revealed 10 cysteine residues, which are conserved among human, mouse and rat (Fig. 5A). Nine of ten cysteine residues locate in the DNA-binding domain (DBD) of PPAR $\gamma$ 1. LC-MS/MS was used to identify the specific cysteine residues undergoing S-nitrosylation. Biotin-maleimide modified cysteines represented S-nitrosylated sites. As shown in Fig. 5B–C, Cys109 and Cys168 were found to be S-nitrosylated on mouse PPAR $\gamma$ 1 by GSNO *in vitro*. To further validate whether these sites were S-nitrosylated *in vivo*, CMX-mPPAR $\gamma$ 1 with Cys109 or (and) Cys168 changed to alanine (C109A, C168A and 2 C-A) were constructed and transfected into HeLa cells. IBP showed that the mutation at Cys168 but not Cys109 abolished the nitrosylation by GSNO (Fig. 5D). It was suggested that S-nitrosylation modified PPAR $\gamma$  predominantly at Cys168 *in vivo*.

### 3.4. S-nitrosylation diminished the transcriptional activity of PPAR $\gamma$

To investigate the functionality of the PPAR $\gamma$  S-nitrosylation, luciferase reporter assay was performed with wild type or the C168A mutant PPAR $\gamma$  transfection. As shown in Fig. 6, GSNO decreased the reporter activity of wild type PPAR $\gamma$  under basal and rosiglitazone-stimulated conditions. However, the suppressive effect of GSNO was not observed on the PPAR $\gamma$  mutant (C168A). Hence, the inhibitory effect of GSNO on PPAR $\gamma$  transcriptional activity was largely attributed to S-nitrosylation at Cys168.

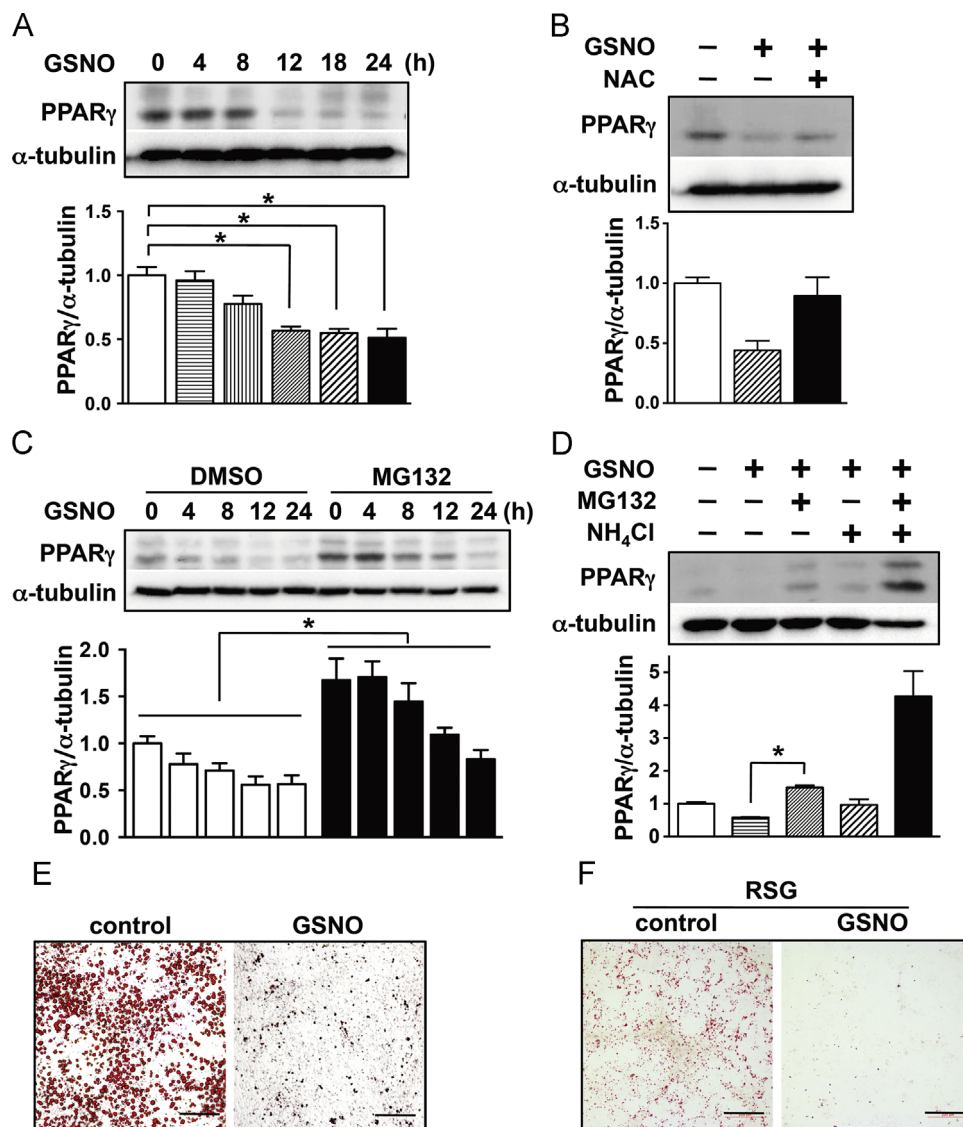
### 3.5. Prolonged exposure to GSNO decreased PPAR $\gamma$ stability and blocked adipogenic differentiation

In order to investigate the chronic effect of PPAR $\gamma$  S-nitrosylation, 3T3-L1 cells were treated with GSNO for up to 24 h. Western blotting showed that the protein level of PPAR $\gamma$  was decreased significantly in 3T3-L1 cells that were exposed to GSNO for more than 12 h (Fig. 7A). Prolonged treatment with GSNO slightly decreased cell viability, but the difference was not statistical significant (Supplementary Fig. 1B). NAC treatment before GSNO rescued the decrease of PPAR $\gamma$  protein (Fig. 7B). Pretreatment with MG132, a proteasome inhibitor, partially prevented the decrease of PPAR $\gamma$  (Fig. 7C). The lysosome inhibitor NH $_4$ Cl did not alleviated PPAR $\gamma$  decline significantly (Fig. 7D). The results suggested the proteasome-dependent degradation might account for the impaired PPAR $\gamma$  stability.

Since PPAR $\gamma$  is an essential transcription regulator for adipogenesis, we examined the potential effect of GSNO on adipogenic differentiation. Primary preadipocyte was induced with insulin, IBMX and dexamethasone for 8 days in the presence or absence of GSNO. Oil red O staining showed that GSNO abrogated adipogenic differentiation (Fig. 7E). Similarly, GSNO largely inhibited the rosiglitazone-stimulated adipogenesis in 3T3-L1 cells (Fig. 7F). Taken together, these results suggested that prolonged exposure to GSNO further impaired PPAR $\gamma$  protein stability and its adipogenic activity.

### 3.6. Macrophage infiltration and suppression of PPAR $\gamma$ activity in visceral adipose tissue (VAT) of db/db mice

Given the *in vitro* results that macrophages stimulated the S-nitrosylation and the exposure to nitrosative stress led to the impaired activity and degradation of PPAR $\gamma$ , we further examined the relationship between pro-inflammatory macrophages and PPAR $\gamma$  in visceral adipose tissue (VAT) obese diabetic db/db mice. Immunostaining of F4/80 revealed severe macrophage infiltration



**Fig. 7.** Extended exposure to GSNO decreased PPAR $\gamma$  protein level and blocked adipogenic differentiation. (A) 3T3-L1 cells were treated with GSNO for indicated times and analyzed by Western blotting using antibodies against PPAR $\gamma$  and  $\alpha$ -tubulin. (B) 3T3-L1 cells were pretreated with NAC (10 mM, 2 h) and then GSNO (1 mM, 12 h). PPAR $\gamma$  protein level was analyzed. (C) After pre-treatment with or without MG132 (10  $\mu$ M, 2 h), 3T3-L1 cells were exposed to GSNO for indicated times and analyzed for PPAR $\gamma$  protein expression. (D) 3T3-L1 cells were treated with MG132 (10 M) or (and) NH $_4$ Cl (20 mM) for 2 h before GSNO (1 mM, 12 h) treatment. PPAR protein level was detected. (E) Rat epididymal preadipocytes were treated with differentiation cocktail in the presence or absence of GSNO. (F) Confluent 3T3-L1 cells were treated with RSG (10  $\mu$ M) or DMSO for 3 days with or without GSNO (1 mM, 1 day) pretreatment. The adipogenic differentiation was assessed with Oil Red O staining. Scale bar: 200  $\mu$ m.

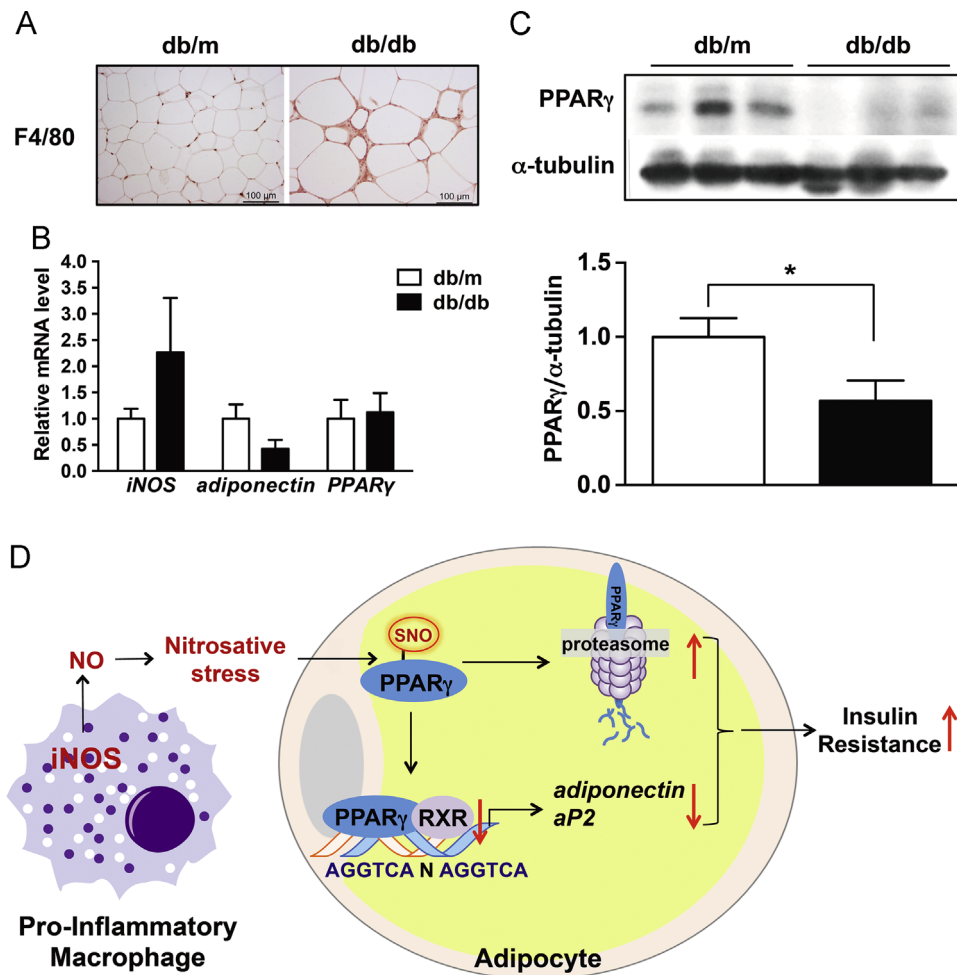
in *db/db* mice (Fig. 8A). Meanwhile, gene expression of *iNOS* was increased in *db/db* mice while the PPAR $\gamma$  target gene *adiponectin* were decreased (Fig. 8B). In primary adipocytes from VAT of *db/db* mice, *adiponectin* and *aP2* were also reduced by LPS-stimulated Raw264.7 macrophages in an *iNOS*-dependent manner (Supplementary Fig. 2A–B). Immunoblotting showed that PPAR $\gamma$  protein level was greatly decreased whereas the mRNA level was not reduced in VAT from *db/db* mice compared with those from *db/m* mice (Fig. 8B, C). These results suggest a close link between the pro-inflammatory macrophage-derived nitrosative stress and impaired PPAR $\gamma$  function in diabetes.

#### 4. Discussion

In the present study, we found that pro-inflammatory macrophages negatively regulated the transcriptional activity and protein stability of PPAR $\gamma$  in adipocytes. The S-nitrosylation of PPAR $\gamma$  at the cysteine 168 residue via the *iNOS*-derived NO mediated this

effect. PPAR $\gamma$  S-nitrosylation impaired its binding ability to the promoters of target genes and, hence, the ligand induced expression.

In obese adipose tissue, *iNOS*-derived NO is an inflammatory mediator secreted by pro-inflammatory macrophages that impairs local adipocyte function [3,29]. Increased *iNOS* expression [30] or plasma NO metabolites [31,32] correlated with obesity in obese mouse models or individuals. Here we reported that NO from macrophage *iNOS* reduced PPAR $\gamma$  activity and *adiponectin* expression in adipocytes (Fig. 1C). This result provides new evidence that macrophage-derived NO is a culprit for adipocyte dysfunction in obese diabetes. Further study revealed that NO-induced increased S-nitrosylation of PPAR $\gamma$  was responsible for the reduced *adiponectin* expression, indicating the impaired insulin signaling. Increased NO or protein S-nitrosylation was also reported to affect insulin action. In *db/db* or *ob/ob* obese diabetic mice, diet-induced obesity and liver-specific *iNOS* transgenic mice, increased S-nitrosylation repressed insulin receptor  $\beta$  (IR $\beta$ ) and Akt activity, or insulin receptor substrate-1 (IRS-1) expression, blunting the



#### S-nitrosylation of PPARγ: A potential link between inflammation and insulin resistance.

**Fig. 8.** Macrophage infiltration and suppression of PPARγ activity in visceral adipose tissue (VAT) of *db/db* mice. Epididymal adipose tissues were taken from *db/db* or *db/m* mice. (A) Immunohistochemical stainings for F4/80 were performed on the paraffin-embedded sections. Scale bar: 100 μm. (B) qRT-PCR analysis of *iNOS*, *adiponectin*, *aP2* and *PPARγ* gene expression ( $n=6$ ). (C) Protein levels of PPARγ and α-tubulin were detected with Western blotting. The bar graphs show mean  $\pm$  SEM ( $n=6$ ). \* $p < 0.05$ . (D) Proposed model summarizing the mechanism underlying the cross-talk between pro-inflammatory macrophages and adipocytes with emphasis on the NO-mediated post-translational modification of PPARγ via S-nitrosylation.

insulin action in skeletal muscle and liver [33–35]. NO donors also impaired anti-lipolytic action of insulin by S-nitrosylation of phosphodiesterase 3B (PDE3B) in cultured adipocytes or mice [36]. Here, our finding that suppression of PPARγ by S-nitrosylation provides a new mechanism of insulin resistance caused by macrophage-derived NO during obesity and obese diabetes. However, pro-inflammatory macrophages also secreted several other pro-inflammatory factors such as IFNγ. These pro-inflammatory cytokines also impair the adipocytes' function and contribute to insulin resistance. This notion is supported by the fact that the suppressive effect of pro-inflammatory macrophages on the PPARγ target gene in adipocytes was largely attenuated but not fully abolished by the iNOS inhibitor (Fig. 1C, D).

In this study, we revealed the repression of PPARγ by NO, which was supported by decreased PPARγ transcriptional activity and protein level (Figs. 1, 2, 7). Consistently, several studies had indicated that NO signaling might regulate PPARγ function. In 3T3-L1 cells, NO donors decreased PPARγ promoter activity on adiponectin gene [37] and DNA-binding activity [24]. In adipose tissue of iNOS-deficient mice, PPARγ protein was increased, indicating the decrease of PPARγ protein level by NO. Furthermore, under a high fat diet, the insulin sensitizing effect of rosiglitazone was enhanced in these mice [38]. These observations implied a

causative relationship between nitrosative milieu and the impaired PPARγ function in adipocytes.

S-nitrosylation is sensitive to redox state. S-nitrosylation is sensitive to redox state. GSNO and thioredoxin/thioredoxin reductase (Trx/TrxR) are major systems to denitrosylate protein S-nitrosylation [39,40]. It was reported that deletion of thioredoxin-interacting protein (Txnip, or VDUP1, TBP-2), a protein inhibiting Trx activity [41,42], enhanced PPARγ activity and the rosiglitazone-stimulated adipogenesis, preserving insulin sensitivity [43]. These results indicated that denitrosylation protected PPARγ function, corroborating that PPARγ function is impaired by S-nitrosylation.

We found that S-nitrosylation inhibits PPARγ through two distinct mechanisms. Firstly, short term exposure to GSNO caused the S-nitrosylation of PPARγ leading to decreased transcriptional activity, as evidenced by a reduced activation of PPARγ target genes adiponectin and aP2 (Figs. 1, 2) as well as the PPARγ reporter activity. This was likely due to the location of the thiol-modified cysteine within the DBD of PPARγ (cysteine 168) [44]. Therefore, the S-nitrosylation decreased the ligand-induced PPARγ binding to the target genes (Fig. 3). In fact, S-nitrosylation of the cysteine residues within DBD in several other transcription factors including NFκB, androgen receptor, MEF2 and c-myc has been

documented to repress their transcriptional activities [44–47]. Thiol-modification was known to disrupt the conformational flexibility of the DBD [46,47]. On the other hand, extended exposure to nitrosative stress further impaired the protein stability of PPAR $\gamma$  (Fig. 7A). This could be attributed to the accelerated degradation of PPAR $\gamma$  in proteasome because the proteasome inhibitor MG132 partially rescued the GSNO induced degradation (Fig. 7C). It is worth noting that lysosome pathway might also be involved in this process since the degradation was prevented in the presence of MG132 and NH<sub>4</sub>Cl, a lysosome inhibitor (Fig. 7D). As a de-nitrosylating agent, NAC decreased the S-nitrosylation of PPAR $\gamma$  (Fig. 4B) and the PPAR $\gamma$  degradation induced by GSNO (Fig. 7B), supporting a role of S-nitrosylation in decreasing the PPAR $\gamma$  stability.

*Db/db* mouse is featured with severe obesity and insulin resistance, thus considered as a rodent model for human type 2 diabetes. We demonstrated that pro-inflammatory macrophages suppressed *adiponectin* and *aP2* expression in primary adipocytes from *db/db* mice in an iNOS-dependent manner (Supplementary Fig. 2). We also showed that increased accumulation of macrophages, induced iNOS expression and suppressed *adiponectin* expression in the VAT of *db/db* mice (Fig. 8A, B), indicating a link between nitrosative milieu and decreased PPAR $\gamma$  activity. Surprisingly, PPAR $\gamma$  protein level was significantly decreased compared to the control *db/m* mice (Fig. 8C). A similar decrease in PPAR $\gamma$  protein level was previously reported in adipose tissue from obese women with gestational diabetes [48]. Whether the decreased PPAR $\gamma$  level in VAT of *db/db* mice resulted from the chronic nitrosative stress and accelerated degradation remains to be investigated.

In conclusion, we demonstrated that pro-inflammatory macrophages negatively regulated adipocyte PPAR $\gamma$  activity via S-nitrosylation. These results may provide a novel link between metabolic inflammation and the development of insulin resistance.

## Duality of Interest

No potential conflicts of interest relevant to this article were reported.

## Author Contributions

N.W. and C.C. conceptualized and designed the research; R.Y., Y.L. and Y.L. performed the experiments; R.Y. analyzed the data; Y.L., L.F. and P.X. analyzed the data about Mass Spectrometry. Y.G. and Y.C. contributed to the *in vivo* study. R.Y. and P.X. drafted the manuscript; N.W. and C.C. edited and revised the manuscript; and R.Y., L.F., Y.L., P.X., Y.L., Y.G., Y.C., C.C. and N.W. approved the final version of the manuscript. N.W. and C.C. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in

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